

Listing of the claims.

1. (original) A multiplex PCR method, comprising the step of conducting a PCR amplification on a DNA sample in a PCR reaction mixture, wherein the PCR amplification is conducted in a first amplification stage and a second amplification stage, each amplification stage comprising one or more PCR cycles and each PCR cycle comprises a denaturing step, an annealing step and an elongation step that may be conducted at the same temperature as the annealing step, wherein the PCR amplification of the second amplification stage is conducted under different reaction conditions than the PCR amplification of the first amplification stage to modulate the relative rate of production of a first amplicon by a first primer set and a second amplicon by a second primer set during the first and second amplification stages.
2. (original) The method of claim 1, wherein the second primer set is added to the reaction mixture at the beginning of the second amplification stage.
3. (currently amended) The method of claim 1, wherein one of the first PCR primer set and the second PCR primer set produce one of a  ~~$\beta$ -GUS-specific~~ $\beta$ -glucuronidase-specific amplicon, an 18SrRNA-specific amplicon, a ~~CEA-~~specific carcinoembryonic antigen-specific amplicon and a tyrosinase-specific amplicon.
4. (original) The method of claim 3, wherein one of the first PCR primer set and the second PCR primer set includes a primer comprising the sequence of one of SEQ ID NOS: 3, 4, 6, 7, 11, 12, 13, 14, 16, 17, 19 and 20, or a derivative thereof.
5. (original) The method of claim 3, wherein one of the first PCR primer set and the second PCR primer set includes a primer consisting of one of SEQ ID NOS: 3, 4, 6, 7, 11, 12, 13, 14, 16, 17, 19 and 20, or a derivative thereof.

6. (original) The method of claim 3, wherein one of the first PCR primer set and the second PCR primer set includes a primer consisting of one of SEQ ID NOS: 3, 4, 6, 7, 11, 12, 13, 14, 16, 17, 19 and 20.
7. (original) The method of claim 1, wherein the reaction mixture comprises a DNA sample, the first primer set having a first effective  $T_m$  and the second primer set having a second effective  $T_m$  different from the first effective  $T_m$ , wherein the annealing step of the first amplification stage is conducted at a different temperature than the annealing step of the second amplification stage.
8. (original) The method of claim 7, wherein the annealing step and the elongation step in a PCR cycle of at least one of the first amplification stage and the second amplification stage are conducted at the same temperature.
9. (original) The method of claim 7, wherein the annealing step and the elongation step in a PCR cycle of at least one of the first amplification stage and the second amplification stage are conducted at different temperatures.
10. (currently amended) The method of claim 7, wherein the first primer set produces a  ~~$\beta$ -GUS-specific~~ $\beta$ -glucuronidase-specific amplicon and the second primer set produces a ~~CEA-specific~~carcinoembryonic antigen-specific amplicon, the  $T_m$  of the first primer set being about 10°C lower than the  $T_m$  of the second primer set and the annealing temperature for the PCR amplification of the first amplification stage is about 10°C lower than the annealing temperature for the PCR amplification of the second amplification stage.
11. (original) The method of claim 10, wherein the first PCR primer set consists of SEQ ID NOS: 16 and 17, and the second PCR primer set consists of SEQ ID NOS: 6 and 7, the annealing temperature for the PCR amplification of the first amplification stage is the equivalent of about 53°C and the annealing temperature for the PCR amplification of the second amplification stage is the

equivalent of about 64°C, based on initial primer concentration of both PCR primer sets of 400 nM/L and an effective T<sub>m</sub> for the first PCR primer set of about 50°C and an effective T<sub>m</sub> for the second PCR primer set of about 60°C.

12. (original) The method of claim 1, wherein the denaturation step for each cycle is about 1 second.

13. (original) The method of claim 1, wherein the denaturation step for each cycle is less than about 1 second.

14. (original) The method of claim 1, further comprising the step of conducting a reverse transcription reaction on an RNA sample prior to the first amplification stage, and prior to the addition of PCR primers to the reaction mixture, to produce the DNA of the DNA sample of the reaction mixture.

15. (original) The method of claim 14, wherein the reverse transcription reaction is conducted for less than about 10 minutes.

16. (original) The method of claim 14, wherein the reverse transcription reaction is conducted for about 2 minutes.

17. (original) The method of claim 14, wherein one or both of an Internal Positive Control RNA and an Internal Positive Control DNA is added to the reverse transcription reaction.

18. (original) The method of claim 1, wherein an Internal Positive Control DNA is added to the PCR reaction mixture.

19. (original) The method of claim 18, wherein the Internal Positive Control DNA comprises the sequence of one of SEQ ID NOS 23-25, or a derivative thereof.

20. (original) The method of claim 18, wherein the Internal Positive Control DNA contains one or more uracil residues.
21. (original) The method of claim 1, wherein the amplification stages include quantitative PCR reactions using a fluorescent reporter to indicate accumulation of a specific amplicon.
22. (original) The method of claim 21, wherein the quantitative PCR reactions are fluorescent 5' nuclease assays.
23. (original) The method of claim 21, wherein the fluorescent reporter is a molecular beacon.
24. (original) The method of claim 1, wherein one or more reagents for the reaction mixture are provided for use in the reaction mixture in a cartridge suitably configured for use in an automated system.
25. (original) The method of claim 24, wherein the cartridge is disposable after a single use.
26. (original) The method of claim 24, wherein the cartridge contains additional reagents or mechanical components compartmentalized separately or together with the reagents for the reaction mixture, the additional reagents or mechanical components adapted for one of cell or tissue lysis, RNA purification and reverse transcription.
27. (original) The method of claim 24, wherein the amplification stages include quantitative PCR reactions using a fluorescent reporter to indicate accumulation of a specific amplicon and the automated system automatically shifts the PCR reaction from the first amplification stage to the second

amplification stage when the fluorescent reporter accumulates in the reaction mixture to a threshold level.

28. (original) The method of claim 1, wherein the first and second stages are conducted sequentially in the same reaction vessel.

29. (original) The method of claim 1, wherein there is expected to be at least about a 30-100-fold difference in the number of target sequences of the first primer set and of the second primer set in the DNA sample.

30. (original) A multiplex PCR method, comprising the step of conducting a PCR amplification on a PCR reaction mixture in a first stage and a second stage, the reaction mixture comprising a DNA sample, a first primer set having a first effective  $T_m$  and a second primer set having a second effective  $T_m$  different from the first effective  $T_m$ , each amplification stage comprising one or more PCR cycles, each PCR cycle comprising a denaturing step, an annealing step and an elongation step that may be conducted at the same temperature as the annealing step, wherein the annealing step of the first amplification stage is conducted at a different temperature as the annealing step of the second amplification stage to modulate the relative rate of production of a first amplicon by the first primer set and a second amplicon by the second primer set during the first and second amplification stages.

31. (original) The method of claim 30, wherein the annealing step and the elongation step in PCR cycles of at least one of the first amplification stage and the second amplification stage are conducted at the same temperature.

32. (original) The method of claim 30, wherein the annealing step and the elongation step in PCR cycles of at least one of the first amplification stage and the second amplification stage are conducted at different temperatures.

33. (original) The method of claim 30, wherein the effective T<sub>m</sub> of the first primer set and the effective T<sub>m</sub> of the second primer set differ by at least about 5°C.

34. (currently amended) The method of claim 30, wherein one of the first PCR primer set and the second PCR primer set produce one of a ~~β-GUS-specific~~β-glucuronidase-specific amplicon, an 18SrRNA-specific amplicon, a ~~CEA-specific~~carcinoembryonic antigen-specific amplicon and a tyrosinase-specific amplicon.

35. (original) The method of claim 34, wherein one of the first PCR primer set and the second PCR primer set includes a primer comprising the sequence of one of SEQ ID NOS: 3, 4, 6, 7, 11, 12, 13, 14, 16, 17, 19 and 20, or a derivative thereof.

36. (original) The method of claim 34, wherein one of the first PCR primer set and the second PCR primer set includes a primer consisting of one of SEQ ID NOS: 3, 4, 6, 7, 11, 12, 13, 14, 16, 17, 19 and 20, or a derivative thereof.

37. (original) The method of claim 34, wherein one of the first PCR primer set and the second PCR primer set includes a primer consisting of one of SEQ ID NOS: 3, 4, 6, 7, 11, 12, 13, 14, 16, 17, 19 and

38. (original) The method of claim 30, wherein the annealing step of the first amplification stage is conducted at a temperature greater than the annealing step of the second amplification stage.

39. (currently amended) The method of claim 38, wherein one of the first and second primer sets produces one of a ~~β-GUS-specific~~β-glucuronidase-specific amplicon, a ~~CEA-specific~~carcinoembryonic antigen-specific amplicon, an 18SrRNA amplicon and a tyrosinase amplicon.

40. (currently amended) The method of claim 38, wherein the first primer set produces a  ~~$\beta$ -GUS-specific~~ $\beta$ -glucuronidase-specific amplicon and the second primer set produces a ~~CEA-specific~~carcinoembryonic antigen-specific amplicon.

41. (original) The method of claim 30, wherein the annealing step of the first amplification stage is conducted at a temperature less than the annealing step of the second amplification stage.

42. (original) The method of claim 30, further comprising the step of conducting a reverse transcription reaction on an RNA sample prior to the first amplification stage, and prior to the addition of one of PCR primers and a thermostable DNA polymerase to the reaction mixture, to produce DNA in the DNA sample of the reaction mixture.

43. (original) The method of claim 42, wherein the reverse transcription reaction is conducted for less than about 10 minutes.

44. (original) The method of claim 42, wherein the reverse transcription reaction is conducted for about 2 minutes.

45. (original) The method of claim 42, wherein one or both of an Internal Positive Control RNA and an Internal Positive Control DNA is added to the reverse transcription reaction.

46. (original) The method of claim 30, wherein an Internal Positive Control DNA is added to the PCR reaction mixture.

47. (original) The method of claim 46, wherein the Internal Positive Control RNA comprises the sequence of one of SEQ ID NOS 23-25.

48. (original) The method of claim 30, wherein one or more reagents for the reaction mixture are provided for use in the reaction mixture in a cartridge suitably configured for use in an automated system.

49. (original) The method of claim 48, wherein the cartridge is disposable after a single use.

50. (original) The method of claim 48, wherein the cartridge contains additional reagents or mechanical components compartmentalized separately or together with the reagents for the reaction mixture, the additional reagents or mechanical components adapted for one of cell or tissue lysis, RNA purification and reverse transcription.

51. (original) The method of claim 30, wherein the amplification stages include quantitative PCR reactions using a fluorescent reporter to indicate accumulation of a specific amplicon.

52. (original) The method of claim 51, wherein the quantitative PCR reactions are fluorescent 5' nuclease assays.

53. (original) The method of claim 51, wherein the fluorescent reporter is a molecular beacon.

54-58 (cancelled).

59. (original) An RT-PCR method, comprising the steps of:

- (a) conducting a reverse transcription reaction for less than about 10 minutes on an RNA sample in a reaction mixture to produce a DNA sample;
- (b) adding to the reaction mixture a first primer set having a first effective  $T_m$ , a second primer set having a second effective  $T_m$  different from the first effective  $T_m$  and a thermostable DNA polymerase; and



(c) conducting a PCR amplification on the reaction mixture in a first amplification stage and a second amplification stage, each amplification stage comprising one or more PCR cycles and each PCR cycle comprises a denaturing step of about 1 second or less, an annealing step of less than about 10 seconds and an elongation step of less than about 10 seconds that may be conducted at the same temperature as the annealing step, wherein the annealing step of the first amplification stage is conducted at a lower temperature than the annealing step of the second amplification stage to modulate the relative rate of amplification of a first target sequence by the first primer set and a second target sequence by the second primer set during the first and second amplification stages,

wherein first target sequence is expected to be at least about 30-fold more prevalent in the DNA sample than the second target sequence.

60. (currently amended) A method for increasing sensitivity and specificity of a one-tube RT-PCR method, comprising the steps of:

- (a) conducting a reverse transcription reaction for less than about 10 minutes on an RNA sample in a reaction mixture to produce a DNA sample;
- (b) adding a PCR reagent composition containing a PCR primer set and a thermostable DNA polymerase to the reaction mixture; and
- (c) conducting a PCR amplification on the reaction mixture.

61. (original) The method of claim 60, wherein prior to the PCR amplification, the PCR reagent composition is separated from the reaction mixture in a reaction vessel by a physical barrier which is removed prior to or during the first cycle of the PCR reaction, thereby adding the PCR reagent composition to the reaction mixture.

62. (cancelled).

63. (original) The method of claim 60, wherein the reverse transcription reaction is conducted for about 2 minutes.

64. (original) The method of claim 60, wherein the RT-PCR method is performed in an automated system and the reagents for the RT-PCR method are stored in a cartridge having a plurality of compartments in which the reagents are stored prior to use in the RT-PCR method, wherein the automated system adds the reagents to a reaction vessel from the cartridge according to a programmed sequence.

65. (original) An RT-PCR method comprising the steps of:

- (a) conducting a reverse transcription reaction for less than about 10 minutes on an RNA sample in a reaction mixture; and
- (b) conducting a PCR reaction on the reaction mixture.

66. (original) The method of claim 65, wherein the reverse transcription reaction is conducted for about 2 minutes.

67-77 (cancelled).

78. (previously presented) An intraoperative PCR diagnostic method comprising the steps of:

- (a) obtaining a tissue sample from a patient in an operation; and
- (b) determining by a PCR method performed during the operation if expression of an indicator transcript exceeds a threshold level.

79. (previously presented) The method of claim 78, further comprising the step of continuing the operation in a manner dictated by results of the determining step.

80. (previously presented) The method of claim 78, wherein the tissue sample is a tumor biopsy and the PCR method is specific to an indicator transcript that, when the indicator transcript is overexpressed, is indicative of a malignancy.

81. (currently amended) The method of claim 80, wherein the indicator transcript is a CEAcarcinoembryonic antigen transcript.

82. (previously presented) The method of claim 78, wherein the PCR method is a multiplex PCR method, comprising the step of conducting a PCR amplification on a DNA sample in a PCR reaction mixture, wherein the PCR amplification is conducted in a first amplification stage and a second amplification stage, each amplification stage comprising one or more PCR cycles and each PCR cycle comprises a denaturing step, an annealing step and an elongation step that may be conducted at the same temperature as the annealing step, wherein the PCR amplification of the second amplification stage is conducted under different reaction conditions than the PCR amplification of the first amplification stage to modulate the relative rate of production of a first amplicon by a first primer set and a second amplicon by a second primer set during the first and second amplification stages.

83. (previously presented) The method of claim 78, wherein the PCR method is a multiplex PCR method, comprising the step of conducting a PCR amplification on a PCR reaction mixture in a first stage and a second stage, the reaction mixture comprising a DNA sample, a first primer set having a first effective  $T_m$  and a second primer set having a second effective  $T_m$  different from the first effective  $T_m$ , each amplification stage comprising one or more PCR cycles, each PCR cycle comprising a denaturing step, an annealing step and an

elongation step that may be conducted at the same temperature as the annealing step, wherein the annealing step of the first amplification stage is conducted at a different temperature as the annealing step of the second amplification stage to modulate the relative rate of production of a first amplicon by the first primer set and a second amplicon by the second primer set during the first and second amplification stages.

84. (currently amended) The method of claim 78, wherein the PCR method is a PCR method, comprising the step of conducting a PCR amplification, the PCR amplification comprising a plurality of PCR cycles, on a PCR reaction mixture comprising a nucleic acid sample, a primer set in which the concentration of each of the primers of the primer set is at least about 400 nM, each PCR cycle comprising a denaturing step, an annealing step and an elongation step which may be conducted concurrently with the annealing step, wherein the PCR amplification produces one of a  ~~$\beta$ -GUS-specific~~ $\beta$ -glucuronidase-specific amplicon, an 18SrRNA-specific amplicon, a tyrosinase-specific amplicon and a ~~CEA-specific~~carcinoembryonic antigen-specific amplicon.

85. (previously presented) The method of claim 78, wherein the PCR method is an RT-PCR method, comprising the steps of:

- (a) conducting a reverse transcription reaction for less than about 10 minutes on an RNA sample in a reaction mixture to produce a DNA sample;
- (b) adding to the reaction mixture a first primer set having a first effective  $T_m$ , a second primer set having a second effective  $T_m$  different from the first effective  $T_m$  and a thermostable DNA polymerase; and
- (c) conducting a PCR amplification on the reaction mixture in a first amplification stage and a second amplification stage, each amplification stage comprising one or more PCR cycles and each PCR cycle comprises a denaturing step of about 1 second or less, an annealing step of less than about 10 seconds

and an elongation step of less than about 10 seconds that may be conducted at the same temperature as the annealing step, wherein the annealing step of the first amplification stage is conducted at a lower temperature than the annealing step of the second amplification stage to modulate the relative rate of amplification of a first target sequence by the first primer set and a second target sequence by the second primer set during the first and second amplification stages,

wherein first target sequence is expected to be at least about 30-fold more prevalent in the DNA sample than the second target sequence.

86. (previously presented) The method of claim 78, wherein the PCR method is an RT-PCR method comprising the steps of:

(a) conducting a reverse transcription reaction for less than about 10 minutes on an RNA sample in a reaction mixture; and

(b) conducting a PCR reaction on the reaction mixture.

87-104 (cancelled).